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Micropropagation of *Hypericum erectum* Thunberg by using Thidiazuron

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ABSTRACT : The effect of plant growth regulators was investigated on *in vitro* shoot proliferation from axillary bud explants of *Hypericum erectum*. To determine the optimal cytokinin for proliferation of axillay buds, we carried out screening four cytokinins (BA, kinetin, 2iP, TDZ). When nodal segments were cultured on MS medium supplemented with 4.5 μM TDZ (thidiazuron), a number of shoots were induced. Our results indicated that the addition of TDZ to culture medium resulted in the induction of significantly more axillary buds than in the addition of other cytokinins. The optimal concentration of TDZ for proliferation of axillary buds was 10 μM. 92% of shoots spontaneously rooted without any plant growth regulator (PGR) and formed whole plantlets within one month. More than 95% of these regenerants survived and they did not show any detectable variation in morphology or growth characteristics compared to their donor plants.

Key words : axillary bud, cytokinin, *Hypericum erectum,* thidiazuron

INTRODUCTION

Hypericum erectum, which belongs to the Hypericaceae family, has been used for the treatment of bleeding, a bruise, and a tumor in Korea since ancient times. There are several *Hypericum* species such as, *H. erectum*, *H. ascyron*, *H. laxum*, *H. oliganthum*, *H. attenuatum*, and *H. conferissimum* in Korea and *Hyperium perforatum* is well known as St. John's wort in the west world. St. John's wort has been used for the treatment of neurological disorders and depression (Alan & Miller, 1998), and for the treatment of cancers as anti-cancer compounds (Schempp *et al.*, 2002). St. John's wort contains hypericin, pseudohypericin and hyperforin as the major compounds. *Hypericum erectum*, which is a medicinal plant used in Korea, also contains these all compounds. It has been found that the content of these compounds in *H. erectum* is 1.45-times higher than those in St. John's wort (Kim *et al.*, 2005).

In vitro propagation offers many advantages over conventional propagation methods. True-to-type multiplication provides uniform plants with genetic identity. Morphological and chemical uniformity among plants regenerated by this technique has been reported in various species of medicinal plants, such as shoot-tip and axillary bud cultures of *Aconitum carmichaeli* (Hatano *et al.*, 1988) and *Kalopanax pictus* (Kim *et al.*, 2002), shoot-tip cultures of *Atractylodes* species (Hatano *et al.*, 1990), and node cultures of *Gentiana scabra* (Yamada *et al.*, 1991). Our investigations are aimed at applying plant tissue culture techniques to generate high-quality somaclones of *H. erectum* with an increased, constant levels of hypericin and hyperforin for phytomedicine production. Although a shoot multiplication protocol for St. John's wort has been established (Murch *et al.*, 2000), the effectiveness of cytokinins on multiple shoot formation has not yet been demonstrated with *H. erectum*. The present investigation describes the *in vitro* propagation of *H. erectum* through axillary bud multiplication with several cytokinins.

MATERIAL AND METHODS

Plant materials

Seeds of *Hypericum erectum* were obtained from Hamyang Medicinal Plant Experimental Station in Korea. Seeds were sterilized for 10 min with a 3% sodium hypochlorite solution containing 0.1% Tween 20, then rinsed four times with sterile distilled water. Seeds were cultured on MS basal medium (Murashige & Skoog, 1962) supplemented 3% sucrose and 0.45% gelrite (Sigma) for 4 weeks. Seedling was started two weeks after inoculation and then cultured until 3 ㎝ length of

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shoot in petridish containing 30 ㎖ of MS medium and then transferred into a culture bottle (7 × 13 ㎝) containing the same medium. The plants were subcultured every 4 weeks until the beginning of the experiments.

Explants and plant growth regulators

Nodal segments containing two axillary buds were obtained from the aseptically grown plant of *H. erectum*. Plants were divided into 1.0 to 1.5-㎝-long segments containing two axillary buds and each explant was cultured on test media. The effects of cytokinins added to MS medium were tested in the following factorial design: TDZ from 0.45 to 20 μM, kinetin from 0.46 to 23 μM, BA at 4.6 μM, 2iP at 4.9 μM. Bud and shoot numbers were counted after 5 weeks. All media contained 3% sucrose and 0.45% gelite, and the pH was adjusted to 5.8 before autoclaving. Cultures were maintained at 25 ℃ under a 16/8 hr (light/dark) photoperiod with a light intensity of 3,000 lux. For each treatment, a total of three replications each with 14 explants were inoculated and statistical analysis was carried out according to ANOVA. The results were classified according to Ducan's multiple-range test.

Acclimatization and field experiment

After shoot elongation and rooting on PGR-free MS medium supplemented 3% sucrose and 0.45% gelrite, regenerated plants were transferred to small pots containing sand, peat and perlite [3 : 3 : 1(v/v)] under greenhouse conditions.

RESULTS AND DISCUSSION

Murch *et al.* (2000) have reported the development of an *in vitro* regeneration system that utilizes TDZ for the induction of *de novo* shoots on etiolated hypocotyls segments of *H. perforatum* seedlings. But this protocol has been not applied at *H. erectum*. Alternatively, we attempted axillary bud proliferation for shoot multiplication of *H. erectum* by using several cytokinins.

Seeds began to germinate after two weeks, with the maximum germination (85%) after three weeks. After germinating of seeds, nodal segments were obtained from several plants of *H. erectum*. When nodal segment was cultured on MS medium supplemented with 5 μM TDZ, a number of shoots were induced as shown in Fig. 1. This experiment led us to examine the effect of several cytokinins on axillary bud proliferation. To determine the optimum cytokinin to proliferate multiple-shoots, we carried out screening cytokinins such as BA, kinetin, 2iP, and TDZ. As shown in Fig. 2, the highest shoot formations were obtained with TDZ and kinetin (8.0 and 4.3 shoots per explant, respectively). The dose of TDZ is known

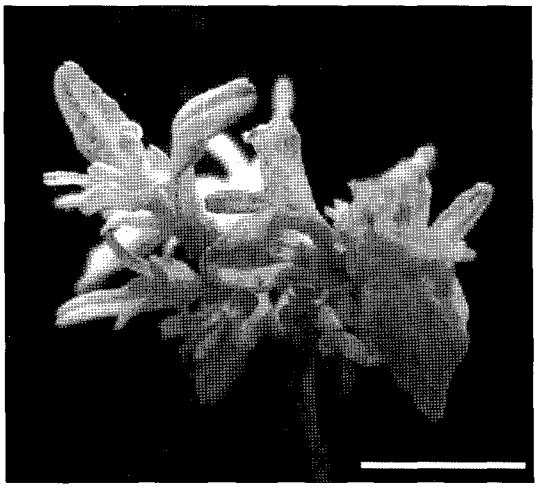

Fig. 1. Induction of multiple shoots from *H. erectum* axillary buds cultured on MS medium supplemented with 4.5 μM TDZ. Bar: 10 mm.

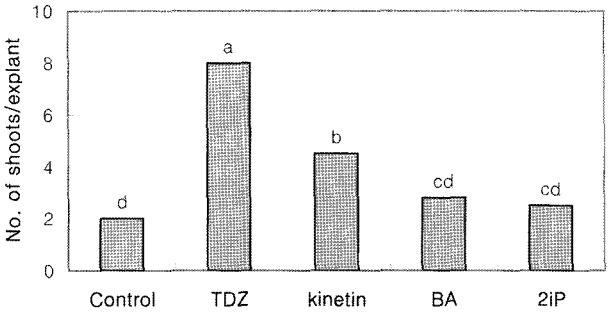

Fig. 2. Shoot production after 5 weeks from axillary buds grown on MS medium with three different cytokinins: free-cytokinin, TDZ (4.5 μM), kinetin (4.6 μM) BA (4.4 μM), and 2iP (4.9 μM). Values over columns with different letters are significantly different at $P \leq 0.05$ (n = 14).

to be critical in shoot organogenesis (Chalupa, 1988; Fasolo *et al.*, 1989; Lu, 1993; van Nieuwkerk *et al.*, 1986; Yusnita *et al.*, 1990). Its biological activity is usually higher than or similar to that of the most active adenine-type cytokinins (Mork *et al.*, 1987; Huetteman & Preece, 1993), but its biochemical action is not completely understood. Our results indicated that the addition of TDZ to culture medium resulted in the induction of significantly more axillary buds than obtained with other cytokinins.

We subdivided the concentration of TDZ and kinetin from 0.45 μM to 20 μM to examine the optimum production of axillary buds (Table 1). When 10 μM TDZ added to culture medium, the highest axillary bud production was observed. The effects of TDZ concentration were highly significant on axillary bud and shoot formation. The optimal concentration of TDZ for proliferation of axillary buds was 10 μM, and increasing the TDZ concentration to levels higher than 20 μM did not

Table 1. The effect of two cytokinins on multiple-shoots induction of *H. erectum*.

Cytokinin	Concentraion (μM)	Mean no. of axillary buds/explant†	Mean no. of shoots/explant†
Control	0	8.1e	2.1e
TDZ	0.5	14.9c	3.7e
	4.5	26.6b	8.5b
	10	32.5a	12.6a
	20	20.4bc	5.5de
Kinetin	0.5	11.9de	2.5e
	4.6	14.2c	4.1de
	11.5	18.3bc	7.4bc
	23	22.5b	8.5b

†Values within a columns with different letters are significantly different at $P \leq 0.05$ (n = 14).

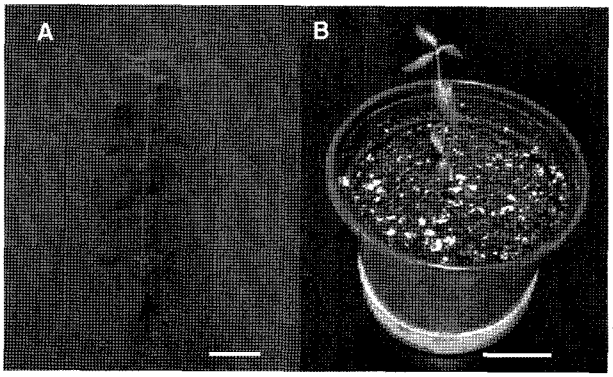

Fig. 3. Root formation from induced shoots of *H. erectum* and acclimatization. A. A plantlet produced from a axillary bud via *in vitro* rooting at 4 weeks. Bar: 2 ㎝. B. A acclimatized plantlet 6 weeks after transfer to the soil. Bar: 4 ㎝.

significantly affect the production of axillary buds. In general, a low range of concentrations of TDZ from 1 nM to 10 μM have been recommended for shoot proliferation (Huetteman & Preece, 1993). High levels of TDZ cause abnormalities or were toxic (Kim *et al.*, 1997; Chand *et al.*, 1999). Additionally, addition of IAA to the TDZ-containing medium did not significantly induce the number of axillary buds (Data not shown).

Culture with TDZ resulted in abnormal leaf morphology, compact shoots and occasional necrosis of *H. erectum* tissue. The problem with shoot elongation was overcome by transferring shoot cultures to a shoot-proliferating medium lacking TDZ and there were no significant differences in morphological characteristics in comparison with control. 92% of shoots spontaneously rooted without any plant growth regulators and formed whole plantlets within 1 month (Fig 3A). The regenerated plants were transferred into a pot containing soil mixture under greenhouse conditions for 6 weeks (Fig 3B). More than 95% of these regenerants survived and they did not show any detectable variation in morphology or growth characteristics compared to their donor plants.

We established a protocol for the micropropagation of *H. erectum* by using thidiazuron. Our results of this investigation clearly show that nodal segments are capable of producing multiple shoots *in vitro*, which can then be rooted to form complete plantlets. This system will be utilized for the useful compounds of *H. erectum* and will also allow possibility of genetic engineering for improving medicinal content.

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